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Award Number: DAMD17-01-1-0234

TITLE: Regulation of Apoptosis by AFG3L2, a Potential Oncogene

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REPORT DATE: August 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command

Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;

Distribution Unlimited

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REPORT DOCUMENTATION PAGE

Form Approved OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

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13. Abstract (Maximum 200 Words,	l (abstract should contain no pi	roprietary or confidential in	formation)		
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apoptotic inhibition. One central casp	pase regulatory molecule, Apaf-1	, binds to and activates ca	aspase 9 in a	large macromolecular structure	
called the apoptosome. In our origina	al proposal, we proposed to diss	ect the apoptotic effects of	f an Apaf-1 b	nding molecule, AFG3L2, a	
putative oncogene that is overexpress	sed in breast and colon cancer of	cells. To determine the eff	ect of AFG3L	2 on apoptosome regulation,	
we developed an assay to test the effect of putative regulators on caspase 9 activation. Although as of yet we have been unable to detect any effect of AFG3L2 on apoptosome function, we were able to demonstrate that an activated MAP Kinase pathway potently					
inhibits apoptosome function. We fou	tosome function, we were able	to demonstrate that an act	tivated MAP I	inase pathway potently	
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apoptosis, caspase, cyto	chrome c, Apaf-1, AF	G3L2		15. NUMBER OF PAGES 25	
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19. SECURITY CLASSIFICATION

Unclassified

OF ABSTRACT

18. SECURITY CLASSIFICATION

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17. SECURITY CLASSIFICATION

Unclassified

20. LIMITATION OF ABSTRACT

Unlimited

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Introduction

Apoptosis, or programmed cell death, is a vitally important cellular process that is disregulated in many different human diseases, including some neurodegenerative disorders, stroke, and many cancers, including breast cancer (Reed 1998; Lee, Zipfel et al. 1999). Because most chemotherapeutic agents act by inducing apoptosis in the target tissue, it is vitally important to understand apoptotic regulation in order to design more effective chemotherapies.

The primary machinery of apoptosis is a family of cysteine proteases called caspases (Cryns and Yuan 1998). These molecules are normally present in the cell as inactive zymogens, and are activated by proteolysis in response to apoptotic stimuli. Some caspases, such as caspase 8, reside on the cell surface and become cleaved as part of the primary response to pro-apoptotic cell-surface ligands such as Fas ligand and TNF alpha (Peitsch and Tschopp 1995), whereas other more downstream caspases, such as caspase 3, are activated by diverse stimuli, including multiple upstream caspases (Li, Nijhawan et al. 1997; Muzio, Salvesen et al. 1997; Stennicke, Jurgensmeier et al. 1998).

One common hallmark of many types of apoptosis is the release of cytochrome c from the mitochondrial intermembrane space (Earnshaw 1999). The exact mechanism governing the release of cytochrome c is controversial, but its role in activating downstream caspases is well understood. After cytochrome c has been released into the cytoplasm, it forms a multimeric complex with Apaf 1, a 130 kDa ATPase. It is thought that dATP hydrolysis changes the conformation of the Apaf 1 caspase recruitment site (known as the CARD domain-- Caspase Recruitment Domain), and that cytochrome c stabilizes this conformation (Zou, Li et al. 1999). Caspase 9 molecules then bind to the activated Apaf-1 molecules through their CARD domains (Qin, Srinivasula et al. 1999). Once assembled on the Apaf-1 scaffold, caspase 9 is able to cleave and activate other caspase 9 molecules within the Apaf-1/caspase 9 complex. This complex, containing Apaf-1 and active caspase 9, is frequently referred to as the apoptosome.

The immediate downstream target of caspase 9 is caspase 3. Caspase 3 cleaves a large number of substrates *in vivo* and *in vitro*, including nuclear lamins and ICAD (an inhibitor of a DNAse), and its activity correlates with many of the phenotypic hallmarks of apoptosis (Porter and Janicke 1999). Caspase 3 can also be cleaved directly by caspase 8, although in many cell types this effect is amplified by release of cytochrome c (Kuwana, Smith et al. 1998).

Because of the central role caspases 3 and 9 play in the execution of the apoptotic program, studying apoptosome assembly and function will give us great insight into apoptotic regulation, as well as provide a potential chemotherapeutic target for future chemotherapies. We therefore decided to perform a 2-hybrid screen using the N-terminal 531 amino acids of Apaf-1 as bait. One molecule, AFG3L2, was pulled out of the screen multiple times. Our preliminary results indicated that AFG3L2 was overexpressed in breast, lung, and colon tumors. These data, in combination with the fact that AFG3L2 binds to Apaf-1, a central apoptotic regulator, indicate that AFG3L2 is a potential oncogene that may inhibit apoptosis in cancer cells. This report seeks to delineate the steps we have taken to characterize this novel oncogene and explore its effects on apoptosome function.

Experimental Results and Discussion

We chose to focus initially on Technical Objective 2, which was to examine possible mechanisms for AFG3L2-mediated inhibition of apoptosis. Because we had already demonstrated that AFG3L2 binds to Apaf-1, we hypothesized that AFG3L2 might bind to and directly inhibit the apoptosomal machinery. In our proposal, we detailed plans to test AFG3L2's effect on *in vitro* caspase 9 processing using the *in vitro* caspase 9 processing reaction described by Zou and colleagues (Zou, Li et al. 1999). However, we were unable to replicate those results in our laboratory, possibly because

the highly recombinogenic nature of the Apaf-1 cDNA (personal communication, G. Nunez) makes cloning and purification of large quantities of recombinant Apaf-1 a more difficult task than was anticipated. Therefore, we chose to develop an alternative method to assay caspase 9 processing and Apaf-1 function; we could then use this alternative protocol to assay the effect of AFG3L2 and other potential apoptotic regulators on apoptosome function.

It has been observed that crude extracts made from eggs of hormonally treated *Xenopus laevis* can spontaneously reconstitute *in vivo* apoptotic processes such as nuclear fragmentation, DNA laddering, and caspase activation *in vitro* (Newmeyer, Farschon et al. 1994). This apoptotic crude extract is rich in protein (about 40 mgs/ml) and retains organelles such as mitochondria. Furthermore, it can be fractionated into cytosolic, light membrane, and heavy membrane fractions; the heavy membrane fraction contains mitochondria and is required for the *in vitro* apoptotic program observed in crude extracts. Because we wanted to ensure that we could purify a large enough quantity of apoptosomal components to reliably monitor apoptosome function, we chose to develop an apoptosome function assay using the highly concentrated *Xenopus* cytosol, which contains more than 30 mgs/ml protein.

Because this cytosolic fraction lacks mitochondria, it does not display any of the phenotypic hallmarks of apoptosis that are observed in the crude extract; however, it is possible to activate an *in vitro* apoptotic program in these extracts by addition of exogenous recombinant cytochrome c (Kluck, Martin et al. 1997). We can then monitor caspase 3 activity using colorimetric substrates (Biomol) (see Figure 1, with legend).

However, this assay does not allow us to distinguish between effects on post-cytochrome c caspase activation and specific effects on apoptosome formation or function. In order to separate out apoptosomes from the rich mix of molecules present in the cytosolic fraction, we attempted to purify the apoptosomal components away from the cytosol using cytochrome c that had been covalently linked to agarose beads (Sigma). We found that these purified apoptosomes can then be assayed for activity against a radiolabeled, *in vitro* translated caspase 9 substrate (see Fig 2, with legend).

We used these assays to evaluate a panel of molecules that have been shown to affect apoptosis in whole cells, including the kinases Mos, MEK, and ERK, which are involved in the Mitogen-Activated Protein (MAP) kinase pathway, and the oncogenic fusion protein p185 BCR-Abl. Although we have not yet observed any effect of AFG3L2 on caspase 3 or 9 activity or on apoptosome activation using these assays, we observed that MAP Kinase pathway activation did, in fact, inhibit cytochrome cdependent caspase 3 activity in the presence of cytochrome c (Figure 3A, 3B, and 3C, with legend). This observation led to further investigation of the role of the MAP kinase pathway in apoptotic inhibition. These and other data were eventually published in the January 2002 issue of Molecular Biology of the Cell (Appendix A) (Tashker, Olson et al. 2002). Using our caspase 9 processing assay (Figure 2), we demonstrated that apoptosomes purified from Mos-treated lysates are less active than those purified from control-treated extracts (Figure 4A, 4B). We were also able to utilize this assay to show that direct phosphorylation by ERK2, but not by the less active ERK1 or Rsk, partially inhibits apoptosome function (Figure 5). We are investigating the role that these kinases may play in synergizing with the putative oncogene AFG3L2.

Key Research Accomplishments

- Publication showing MAPK-dependent inhibition of cytochrome c-mediated caspase 3 activation {Tashker, 2002 #1}
- Development of an assay to analyze apoptosome function in the *Xenopus laevis* egg extract (See Figure 2)
- Demonstration that Mos-mediated MAPK pathway activation inhibits apoptosome function
- Demonstration that apoptosomes formed in the presence of an active MAPK pathway are less active than apoptosomes formed in its absence
- Demonstration of direct ERK2-mediated inhibition of the apoptosome

Reportable Outcomes

- Publication of results in Molecular Biology of the Cell (see Appendix)
- Publication of results as a poster at the 2001 Programmed Cell Death meeting,
 Cold Spring Harbor, NY.

Conclusions

The MAP kinase pathway has been shown to inhibit apoptosis, and this work represents an advance in our understanding of the mechanism of that protection. We have demonstrated conclusively that the MAP kinase pathway can block cytochrome c-mediated caspase activation in the absence of transcription or translation; this work is especially important for breast cancer research when considered in light of the fact that ErbB2, the known breast cancer oncogene, can activate the MAP kinase pathway. We hope that this line of inquiry will yield further results that will shed light on the regulation of apoptosis by these molecules, and we are investigating possible synergy between these kinases and AFG3L2-mediated apoptosome regulation.

Our future work will also focus on the first Technical Objective as described in our original grant application; we hope that studies using tissue culture cells to assay for AFG3L2-mediated effects on apoptosis will be uncovered in this system.

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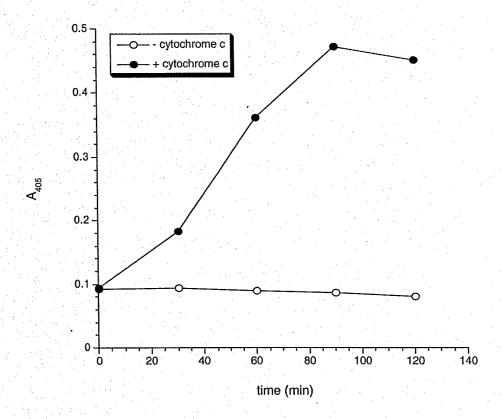


Figure 1. Cytochrome c causes rapid caspase activation in purified *Xenopus* cytosol. Purified *Xenopus* cytosol was combined with ATP regenerating mix and incubated at room temperature with cytochrome c (final concentration 0.7 ng/ul) or buffer control. At the indicated timepoints, aliquots were removed and tested for caspase 3 activity using the model caspase 3 substrate Ac-DEVD-pNA (Biomol), which generates a colored product after cleavage. Caspase 3 activity was then measured by reading the reaction at 405 nm.

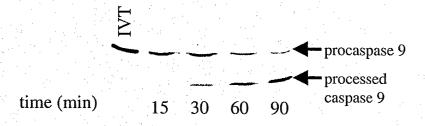


Figure 2. Apoptosome activity assay. Purified apoptosomes are retrieved from purified *Xenopus* cytosol on a cytochrome c-agarose resin (Sigma), washed, and tested for activity against an in vitro translated, radiolabeled caspase 9 substrate (IVT). At the indicated time points, the processing reaction is halted by addition of 2X sample buffer and caspase 9 processing analyzed using SDS-PAGE and autoradiography.

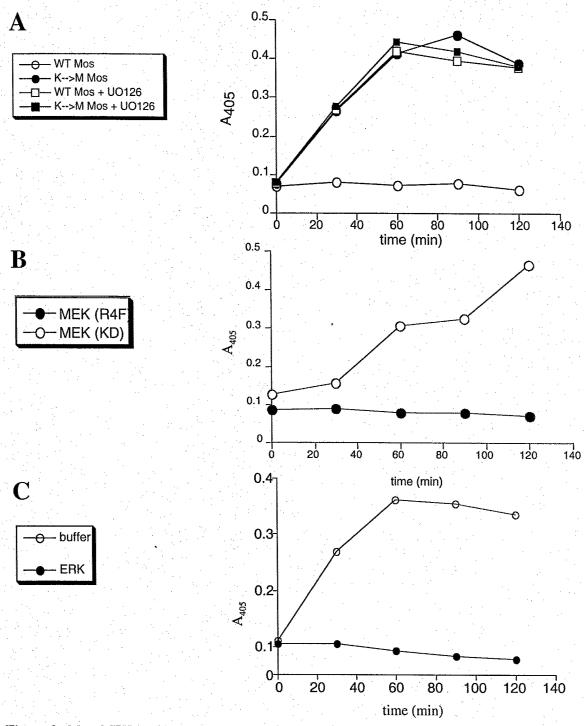
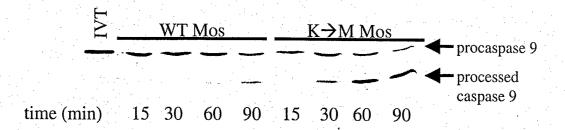


Figure 3. Mos, MEK, and ERK prevent cytochrome c-dependent caspase activation.

(A) Purified Xenopus cytosol was treated for one hour at room temperature with recombinant tagged wild-type Mos(WT Mos) or with kinase dead Mos (K→M Mos), in the presence or absence of 50 μM UO126, a MEK inhibitor. Purified cytochrome c was then added to US or UCSF to a final concentration of 1 μg cytochrome c per 40 mg extract protein. Caspase activity was measured using an AC-DEVD-pNA cleavage assay. (B) Interphase cytosol was treated with recombinant tagged constitutively active MEK (R4F MEK) or kinase dead MEK (KD MEK) for one hour at room temperature. Cytochrome c was then added to 1 μg per 40 mg extract protein. Caspase activity was measured as described above. (C) Interphase cytosol was treated with thiophosphorylated ERK or buffer for one hour at room temperature. Cytochrome c was added and caspase activity measured as described above.







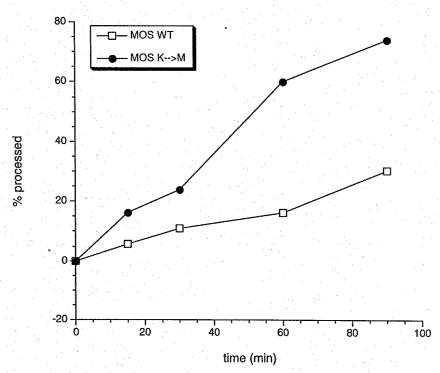


Figure 4. Caspase 9 processing is inhibited in apoptosomes purified from Mos-treated cytosol. (A) Cross-linked cytochrome c beads were incubated in wild-type (WT) or kinase dead (K→M) Mos for one hour at room temperature. Beads were retrieved and washed, and the purified apoptosomes were incubated with processing buffer and *in vitro* translated radiolabeled caspase 9 for the indicated time-points at 30° C. Caspase 9 cleavage was assayed by SDS-PAGE and autoradiography. (B) Caspase 9 cleavage was analyzes using the Phosphorimager system. % processed was calculated as the number of radioactive units in the lower (processed) band, divided by the sum of the units in the lower (processed) band plus the upper (unprocessed) band, times 100

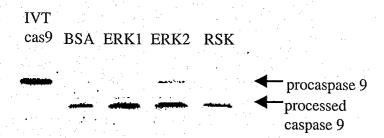


Figure 5. ERK2 partially inhibits caspase 9 processing *in vitro*. Cross-linked cytochrome c beads were incubated in purified Xenopus cytosol for one hour at room temperature. The bound apoptosomes were washed and incubated with ERK1, ERK2, RSK, or BSA (control), in buffer supplemented with 5 mM ATP and 5 mM MgCl₂ for one hour at room temperature. Apoptosomes were washed again and incubated with processing buffer and *in vitro* translated radiolabeled caspase 9 for one hour at 30° C. Caspase 9 cleavage was assayed using SDS-PAGE and autoradiography.

Post-Cytochrome c Protection from Apoptosis Conferred by a MAPK Pathway in *Xenopus* Egg Extracts

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Submitted June 18, 2001; Revised October 31, 2001; Accepted November 14, 2001 Monitoring Editor: Tony Hunter

In response to many different apoptotic stimuli, cytochrome c is released from the intermembrane space of the mitochondria into the cytoplasm, where it serves as a cofactor in the activation of procaspase 9. Inhibition of this process can occur either by preventing cytochrome c release or by blocking caspase activation or activity. Experiments involving in vitro reconstitution of apoptosis in cell-free extracts of *Xenopus laevis* eggs have suggested that extracts arrested in interphase are susceptible to an endogenous apoptotic program leading to caspase activation, whereas extracts arrested in meiotic metaphase are not. We report here that Mos/MEK/MAPK pathways active in M phase—arrested eggs are responsible for rendering them refractory to apoptosis. Interestingly, M phase—arrested extracts are competent to release cytochrome c, yet still do not activate caspases. Concomitantly, we have also demonstrated that recombinant Mos, MEK, and ERK are sufficient to block cytochrome c—dependent caspase activation in purified *Xenopus* cytosol, which lacks both transcription and translation. These data indicate that the MAP kinase pathway can target and inhibit post—cytochrome c release apoptotic events in the absence of new mRNA/protein synthesis and that this biochemical pathway is responsible for the apoptotic inhibition observed in meiotic *X. laevis* egg extracts.

INTRODUCTION

Apoptosis, or programmed cell death, is the process by which superfluous or damaged cells are removed from the body. Apoptotic pathways are widely conserved and have been studied in organisms ranging from flies and worms to humans. The importance of apoptotic cell death to processes such as developmental body patterning, the immune response to viral infection, and the cellular response to damage cannot be underestimated—it has been estimated that >99.9% of the cells generated in the course of a human lifetime die by apoptosis (reviewed in Vaux and Korsmeyer, 1999).

Although a wide variety of stimuli can impinge upon a cell's decision to apoptose, many proapoptotic signals converge on the mitochondria, where they promote release of cytochrome c, an integral respiratory chain protein, from the mitochondrial intermembrane space into the cytoplasm (Green and Reed, 1998). Once released, cytochrome c forms a multimeric complex with Apaf-1, a 130-kDa ATP-binding

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.01–06–0291. Article and publication date are at www.molbiolcell.org/cgi/doi/10.1091/mbc.01–06-0291.

protein (Zou et al., 1999). Thought to stabilize Apaf-1 in its active conformation, cytochrome c renders Apaf-1 competent to recruit the precursor form of one of the "death proteases," caspase 9 (Li et al., 1997; Hu et al., 1999; Jiang and Wang, 2000). Once assembled on the Apaf-1 scaffold, caspase 9 cleaves and activates other procaspase 9 molecules within the Apaf-1/caspase 9 complex (Srinivasula et al., 1998). This multimeric complex, containing Apaf-1, cytochrome c, and active caspase 9, is commonly referred to as the apoptosome (Zou et al., 1999). Once activated within the apoptosome, caspase 9 may then proteolyze and activate other caspases, including caspase 3 (Li et al., 1997), a protease that cleaves a large number of cellular substrates (e.g., nuclear lamins, PARP, the DNAse inhibitor ICAD). These cleavage events are believed to undermine cellular structural integrity and lead to the orderly dismantling of the apoptotic cell (for review see Porter and Janicke, 1999).

Caspase activity is opposed by IAP (inhibitor of apoptosis) proteins. IAPs have been shown to bind and potently inhibit many caspases, including caspases -3, -7, and -9, that are known to act downstream of cytochrome c release (Roy et al., 1997; Deveraux et al., 1998; Deveraux and Reed, 1999). Because these IAPs can block cytochrome c—induced caspase activation, they are potent antagonists of cytochrome c—dependent apoptosis. In turn, IAP function can be antagonized

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then aliquotted, and frozen in liquid nitrogen for future use. All extract components were stored at -80°C .

Production of Mitochondrial Lysates

Frozen mitochondrial pellets were diluted 1:1 in MIB + 25 mM CHAPS on ice for 15 min and then spun through a 0.1- μ m ultrafree-MC filter (Millipore, Bedford, MA) for 15 min at 11,000 rpm in an Eppendorf 5415 C microfuge (Fremont, CA). The filtrate was collected, and protein concentration was measured using the Bio-Rad system (Bio-Rad Protein Laboratories, Hercules, CA).

Immunodepletion Assays

For MEK depletion experiments, Protein A-Sepharose beads (Sigma) were washed in PBS and incubated with anti-MEK antibody (kindly provided by Dr. James Ferrell) for 1 h at 4°C. Bead–antibody complexes were recovered, washed in ELB, and then incubated with 100 μ l crude extract/25 μ l beads. After 1 h at 4°C the antibody–bead complexes were pelleted, and the supernatant was transferred to another tube containing more bead-bound antibody. After a second round of immunodepletion the supernatant was collected and supplemented with ATP-regenerating system (10 mM phosphocreatine, 2 mM ATP, and 150 mg/ml creatine phosphokinase). Extract was then incubated at room temperature and analyzed for caspase 3 activity.

Production of his-tagged Proteins

His-MEK R4F and his-MEK kinase dead constructs in the pRSET vector were all kindly provided by Dr. Tom Guadagno. The plasmids were transformed into the BL21DE3 bacterial strain, grown at 37°C for 2 h, and then induced with 0.4 mM IPTG for 4 h. Bacteria were then pelleted at $6000 \times g$ for 10 min in a Beckman JLA-10.5 rotor, washed in PBS, and then repelleted. Pellets were frozen in liquid nitrogen and stored at -80°C. For protein production, bacteria were resuspended in 12.5 ml lysis buffer (50 mM HEPES, pH 7.7, 750 mM sucrose, 150 mM NaCl, 0.1% Triton X-100) per liter culture, to which had been added 5 mM β -mercaptoethanol, 1 mM PMSF, 5 μM aprotinin and leupeptin, and 0.8 mg/ml lysozyme. Pellets were allowed to lyse on ice for 1 hour, at which point MgCl₂ (final concentration, 20 mM), sodium deoxycholate (final concentration, 0.15%), and DNAse (0.1 mg total) were added, and the lysate was left to incubate on ice until no longer viscous, ~10-20 min. Lysate was centrifuged at 12,000 rpm in a Beckman JS-13.1 rotor for 30 min. Lysate was then poured three times over 300 μ l Ni-NTA agarose (QIAGEN, Santa Clarita, CA) that had been washed in lysis buffer. Bead-bound protein was then washed in 15 ml lysis buffer plus 400 mM NaCl and 20 mM imidazole, then 15 ml lysis buffer alone. For protein elution, beads were eluted with $5 \times 500 \mu l$ lysis buffer plus 200 mM imidazole, then the eluate was concentrated in a Centricon-30 (Millipore), diluted into ELB, then recentriconned to the desired volume. Aliquotted proteins were frozen in liquid nitrogen and stored at -80°C.

Production of MBP-Mos Protein

The plasmids pMALcRI-XE and pMALcRI-XE(KM) (Yew et al., 1992) encoding Xenopus Mos were expressed in the Topp3 bacterial strain, grown 2 h at 37°C, then induced with 0.4 mM IPTG for 2 h at 37°C. Bacteria were pelleted at $6000 \times g$ for 10 min in a Beckman JLA-10.5 rotor, washed in PBS, then repelleted. Pellets were frozen in liquid nitrogen and stored at -80° C. For protein preparation, pellet was resuspended in 25 mls MBP lysis buffer (50 mM Tris, pH 8.0, 50 mM NaCl, 5 mM EDTA) to which had been added 1 mg/ml lysozyme, 5 μ g/ml aprotinin and leupeptin, and 1 mM PMSF. Resuspended bacteria were then lysed by French press. The lysate was centrifuged at $9000 \times g$ for 20 min. The supernatant was removed and run over Q Sepharose resin that had been equilibrated with MBP lysis buffer. Salt was added to the flow-through to reach

a final concentration of 0.5 M NaCl. The flow-through was passed twice over an amylose resin, which was then washed with amylose column buffer (20 mM HEPES, pH 6.8, 88 mM NaCl, 7.5 mM MgCl $_2$) plus 410 mM NaCl, then with amylose column buffer alone. Protein was eluted with 10 \times 1 ml fractions of amylose column buffer plus 10 mM maltose. The fractions with the highest protein concentration as measured by A_{280} were pooled and concentrated using PEG (Sigma), then dialyzed overnight in ELB. Protein was aliquotted and frozen in liquid nitrogen, then stored at $-80^{\circ}\mathrm{C}$.

Caspase 3 Activity Assays

To measure caspase 3 activity, 3 μ l of each sample was incubated with 10 μ l colorimetric substrate AC-DEVD-pNA (Biomol) in Assay Buffer (50 mM HEPES, pH 7.7, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% CHAPS, and 10 mM DTT) for 1 h at 37°C. After a 1-h incubation the reaction was stopped by the addition of 0.2 μ M Ac-DEVD-CHO (Biomol, Plymouth Meeting, PA). Reaction was read at 405 nm with a Labsystems Multiscan Plus plate reader (Fisher Scientific, Pittsburgh, PA).

Cytochrome c Release Assays

For assays in crude extracts the extract was supplemented with ATP regenerating mixture. At various time points cytosolic cytochrome c content was analyzed by diluting 15 μ l crude extract into 15 μ l ELB and filtering diluted extract through a 0.1 μ m ultrafree-MC filter (Millipore). The filtrate was run on 17.5% SDS-PAGE minigels and blotted with anticytochrome c antibody (Cat no. 556433; PharMingen, San Diego, CA).

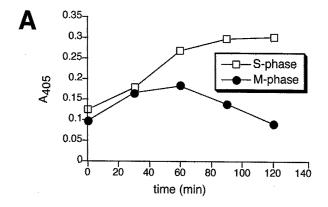
ERK Thiophosphorylation

Recombinant ERK1 (Cat no. 14–188; Upstate Biotechnology, Lake Placid, NY) was thiophosphorylated by diluting 12.5 μ l enzyme (stored in PBS + 50% glycerol) 1:1 with 2× thiophosphorylation buffer (40 mM Tris, pH 7.5, 40 mM MgCl₂, 0.2 mM EDTA, 30 mM β -mercaptoethanol, and 1 mM ATP- γ -S) and incubating with hisMEK immobilized on nickel beads for 4 h at 30°C. As a control, the reaction was also carried out with PBS + 50% glycerol not containing any enzyme. The beads were centrifuged to remove the MEK kinase, and the thiophosphorylated ERK was collected. To remove residual ATP- γ -S, the activated enzyme was diluted out to 500 μ l in ELB and passed through a Microcon YM-10 (Cat no. 42406; Millipore, Bedford, MA) until a >10-fold reconcentration was achieved; this step was repeated three times. ERK activity was measured using recombinant MBP as a substrate.

RESULTS

M Phase Extracts Are Resistant to Apoptosis

As described above, it has been observed that interphase egg extracts are considerably more susceptible to apoptosis than are extracts prepared so as to preserve the meiotic arrest of the egg. To verify this observation, we wanted to compare spontaneous apoptotic activity in extracts stably arrested in M phase (hereafter referred to as CSF extracts, for "cytostatic factor-arrested") and interphase (S) extracts as well as in CSF extracts that had been released into interphase by addition of exogenous calcium (CSF + Ca²⁺). To exclude the possibility that apoptotic inhibition was due to artificial sequestration of calcium by the chelating agent used during CSF extract preparation, EGTA was also added to S extracts (S + EGTA) after Ca²⁺-induced release into interphase; these extracts are unable to return to an M phase state because mitotic cyclins are not present.



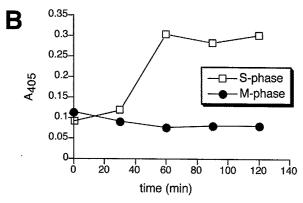


Figure 3. CSF cytosol is resistant to caspase activation. Crude S and CSF extract were further fractionated into membranous and cytosolic fractions. (A) Mitochondrial fractions that had been purified away from the membranous fraction by centrifugation through a percoll gradient were then lysed in a buffer-containing detergent. The lysate was then filtered through a 0.1- μ m microfilter. The filtrate was then added to S or CSF cytosol (US or UCSF, respectively) to a final concentration of 1 mg mitochondrial protein per 60 mg extract protein. Caspase activity was measured as described for Figure 1. Cytochrome c is required for caspase activation by mitochondrial lysate. (B) Purified cytochrome c was added to US or UCSF to a final concentration of 1 μ g cytochrome c per 40 mg extract protein. Caspase activity was measured as described in Figure 1.

CSF, respectively). These reconstituted extracts were then incubated at room temperature and monitored for the development of caspase activity. As shown in Figure 3A, the CSF cytosol was markedly refractory to induction of caspase activity by total mitochondrial protein, although excess mitochondrial protein could overcome this resistance (our unpublished results). In contrast, S cytosol was fully susceptible to caspase activation even by low concentrations of mitochondrial protein. These results indicate that CSF and S phase extracts are differentially sensitive to proapoptotic factors present in the mitochondria, and that cytosolic factors present in CSF extracts can protect extracts from these proapoptotic factors.

It has been shown that exogenous cytochrome c is sufficient to activate caspases 9 and 3 in purified cytosol (Kluck et al., 1997; Li et al., 1997). Because we had demonstrated that cytochrome c is released from mitochondria in CSF extracts

and that CSF extracts are relatively insensitive to the proapoptotic influence of mitochondrial contents, we wanted to determine whether factors within CSF extracts could prevent caspase activation by pure cytochrome c. Therefore, we added purified cytochrome c (Sigma) to S or CSF cytosol (lacking mitochondria) and monitored caspase activity. As shown in Figure 3B, when compared with interphase cytosols, CSF cytosols were markedly resistant to cytochrome c-induced caspase activation.

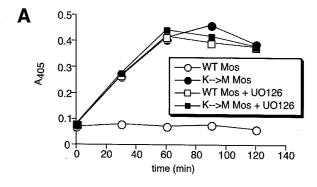
Mos/MEK Kinase Pathway Activity Is Necessary and Sufficient for Apoptotic Inhibition in M Phase Extracts

The most notable difference between CSF and interphase extracts is the presence of high levels of mitotic cyclin/Cdk activity in the former. Indeed, during conversion of mitotic extracts to interphase extracts (or during lysis of eggs in the absence of calcium chelators), the mitotic cdk cdc2/cyclin B is inactivated by calcium-dependent destruction of cyclin B (Watanabe et al., 1991). We therefore assumed that the difference between M and S extracts might lie in the differing levels of cdc2/cyclin B activity. In support of this notion, we found that addition of recombinant cyclin B to interphase extracts could prevent the development of caspase activity (Figure 4A). We were surprised, therefore, when the drug roscovitine, a potent inhibitor of cdc2/cyclin B activity, was unable to promote apoptosis in CSF extracts (our unpublished results). However, because the addition of cyclin B to interphase extracts also activates the MEK/MAP kinase pathway (Guadagno and Ferrell, 1998), which is also highly active in CSF extracts, we hypothesized that a MAP kinase pathway might be responsible for the observed apoptotic inhibition.

The MEK kinase Mos, together with cyclin B, is a primary target of calcium-dependent destruction during the transition from M phase to interphase in the *Xenopus* system (Watanabe *et al.*, 1991). On the basis of our previous results, we hypothesized that continued stimulation of Mos/MEK kinases might be required to block apoptosis in CSF extracts. To test this hypothesis, we immunodepleted endogenous MEK from crude CSF extracts using an anti-MEK antibody. As shown in Figure 4B, immunodepletion of CSF extracts with MEK antibodies, but not control IgG, restored apoptotic activity, indicating that MEK, and, by extension, its activator, Mos, are required to maintain apoptotic inhibition in CSF extracts.

To determine if Mos activation of MEK was sufficient to recapitulate the post–cytochrome c protection from apoptosis observed in CSF extracts, we incubated interphase cytosol with recombinant tagged wild-type Mos (WT Mos) or kinase-inactive Mos (K \rightarrow M Mos) and then added recombinant cytochrome c. Mos kinase activity was sufficient to block caspase activity in the presence of cytochrome c (Figure 5A). This effect was completely reversed by UO126, a MEK inhibitor, indicating that Mos-dependent inhibition of cytochrome c-mediated caspase 3 activity is, as anticipated, mediated through MEK.

We extended these findings by incubating interphase cytosol with recombinant constitutively active (R4F) or kinasedead (KD) MEK and then adding exogenous cytochrome c. R4F MEK alone, but not its kinase inactive variant, was



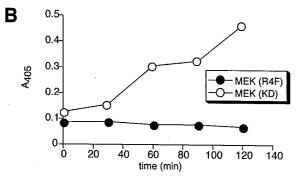


Figure 5. Mos and MEK prevent cytochrome c-dependent caspase activation. (A) Interphase cytosol (US) was treated for one hour at room temperature with recombinant tagged wild-type Mos (WT Mos) or with kinase dead Mos (K \rightarrow M Mos), in the presence or absence of 50 μ M UO126, a MEK inhibitor. Purified cytochrome c was then added to US or UCSF to a final concentration of 1 μ g cytochrome c per 40 mg extract protein. Caspase activity was measured using an AC-DEVD-pNA cleavage assay. (B) Interphase cytosol was treated with recombinant tagged constitutively active MEK (R4F MEK) or kinase dead MEK (KD MEK) for one hour at room temperature. Cytochrome c was then added to 1 μ g per 40 mg extract protein. Caspase activity was measured as described for Figure 1.

Deshmukh and Johnson (1998) have demonstrated that sympathetic neurons are insensitive to microinjected cytochrome c in the presence of growth factors; they propose that a high level of resistance to cytochrome c-induced apoptosis may be necessary for cells such as postmitotic neurons, which are not easily replaced. In Xenopus, the apoptosis-resistant meiotic stage of the cell cycle corresponds to eggs that are en route to being laid or have already been laid. Although these cells can easily be replaced, allowing gametes to apoptose is disadvantageous for organisms such as frogs, which have a low energy investment in their offspring and hence are advantaged by producing the largest possible number of gametes available for fertilization. Alternatively, it may be that apoptotic inhibition is simply a byproduct of the high level of Mos/MEK/MAP kinase activity required to maintain the metaphase II meiotic arrest. In somatic cells, this degree of MAP kinase activation would be observed only after particular signaling events, whereas in the egg this pathway is, by necessity, constitutively active.

Because interphase egg extracts, which no longer have high levels of MAP kinase activity, do not spontaneously

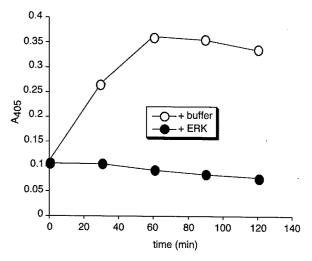


Figure 6. Thiophosphorylated ERK inhibits cytochrome c-dependent caspase activation. Interphase cytosol was treated with thiophosphorylated ERK or equivalently prepared buffer (see MATERIALS AND METHODS) for 30 min at room temperature. Cytochrome c was then added to 1 μ g per 40 mg extract protein. Caspase activity was measured as described for Figure 1.

release cytochrome c until they have been incubated at room temperature for prolonged periods, we assume that there are apoptotic inhibitors operating before cytochrome c release in these extracts (and most likely, in the early fertilized embyros that they mimic). Indeed, it has been suggested that apoptosis is suppressed during the early cleavages in the *Xenopus* embryo (premid blastula transition) by maternally encoded apoptotic inhibitors (Hensey and Gautier, 1997; Stack and Newport, 1997). Although post–cytochrome c protection conferred by MAPK is likely to be lost at fertilization, other, pre–cytochrome c release mechanisms must act to prevent apoptosis during the early embryonic cleavages.

MAP Kinase and the Apoptosome

Once released into the cytosol, the primary function of cytochrome c is to nucleate the apoptosome through recruitment of Apaf-1 and caspase 9. Because our data indicate that MAP kinase targets a post-cytochrome c event, it seems likely that the MAP kinase pathway might target and modulate this initial downstream event, the formation or function of the apoptosome. In theory, apoptosomal inhibition could result from a change in the composition of the apoptosome or from the posttranslational modification (i.e., phosphorylation) of preexisting components. However, the possible targets are not limited to Apaf-1, caspase 9, and cytochrome c; a comparison of apoptosomes isolated from cell lysates with in vitro reconstitutions using purified recombinant components (i.e., caspase 9, cytochrome c, Apaf-1, and dATP) have suggested that apoptosomes from cell lysates may contain additional factors (Cain et al., 2000). Moreover, a number of accessory proteins associated with apoptosomes have been described (e.g., Aven [Chau et al., 2000] and NAC [Chu et al., 2001]). The function of these or other novel molecules may be altered by MAP kinase phosHu, Y., Benedict, M.A., Ding, L., and Nunez, G. (1999). Role of cytochrome c and dATP/ATP hydrolysis in Apaf-1-mediated caspase-9 activation and apoptosis. EMBO J. 18, 3586–3595.

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